

Spring 5-1-2014

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Talbot, Lillian Rose, "The Effect of Repeated Mild Traumatic Brain Injury on Ventricular Volume and Microglial Activation" (2014).
Honors Scholar Theses. 334.
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**The Effect of Repeated Mild Traumatic Brain Injury on Ventricular Volume
and Microglial Activation**

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BS Physiology and Neurobiology with Honors

BS Molecular and Cellular Biology

University of Connecticut Class of 2014

Submitted in Fulfillment of the Honors Scholar Requirement

Acknowledgements

Thank you to Dr. Joanne Conover, for her wonderful mentorship and continued support throughout my two years in her laboratory. My time in the Conover Lab has been instrumental in my decision to pursue a professional career in science. It has been such a fascinating and rewarding experience.

Without the help of Dr. Conover's excellent graduate students, Rebecca Acabchuk, Matt Eastman and Meredith Halling, I would have no project and no idea how to turn on a microscope. Becky, you too have become a trusted mentor and advisee, and I will miss our wonderful conversations.

Thank you to my fellow Conover Lab Undergrads: Andrew Trinh, Ye Sun, Nicholas Gallo, Richard Wolferz, Maha Saleem, May Stern and Yuan Liang. Each of you have inspired me to become a more engaged thinker and dedicated student. You are all wonderful friends, and I truly appreciate your hard work.

Thank you to the UConn Honors Program for helping to enrich my four years as an undergraduate student. Funding for my project was provided by the OUR Research Supplies Grant and the Honors Life Science Thesis Award. I am very grateful for UConn's financial support and commitment to undergraduate research and exploration.

The University of Connecticut has become my home and the backdrop for many of my happiest memories. I will look back on these years fondly, all of my days long.

Abstract

As the leading cause of death and disability in individuals under the age of 45-years-old, Traumatic Brain Injury (TBI) is a public health crisis that demands the attention of the scientific and medical community [28]. The majority of all TBIs that occur in the United States each year are a non-deadly yet detrimental form of closed brain injury known as mild TBI (mTBI) or concussion [6]. Athletes, young people and military personnel all face a high risk of acquiring mTBI as a result of their environments. In our study we have chosen to model repeated mTBI (rmTBI) in the mouse in order to gain a boarder understanding of the mechanisms of injury and cellular changes occurring immediately after and months following rmTBI. Over a 3-month time course, we observed a gradual, yet significant, increase in lateral ventricular volume in mice that had induced rmTBI over that of control mice. In addition we observed changes in microglial morphology and activation throughout the brain immediately after rmTBI and up through 3-months following injury. Both of our findings contribute to the hypothesis that sustaining repeated rmTBIs over time can lead to an increased risk for neurodegeneration or poor recovery.

Introduction

Traumatic brain injury (TBI), an acquired injury resulting from a rapid and sudden blow to the head, is a serious health concern. The CDC estimates that on average 1.7 million people sustain a TBI each year, costing the United States approximately 76.5 billion dollars in direct and indirect medical costs annually [6]. Traumatic brain injury can potentially leave fully functioning individuals handicapped or impaired with little hope for recovery, putting strain on family and caregivers. Young adults, athletes, and military personnel make up a large group of people routinely diagnosed with a non-deadly yet symptomatically significant form of TBI known as mild TBI (mTBI) [3] [6]. Improvements have been made in identifying patients with concussions or mTBI, however treatment options are limited, and little is known about the long-term effects of physical injury to the brain or the brain's capacity for recovery [17]. Physical symptoms of mTBI including headache, dizziness, or memory loss often disappear within a few weeks of the injury [5]. Sustaining repeated mTBI (rmTBI), however, may lead to Chronic Traumatic Encephalopathy (CTE), a severe neurodegenerative disease characterized by diffuse accumulations of hyperphosphorylated tau throughout the brain. Patients diagnosed posthumously with CTE have been as young as 17-years-old [3] [21] [26]; however, the usual age of onset is between 40 and 50 years old.

Serious cognitive decline, short-term memory loss, personality changes, aggression, and suicidal thoughts are all symptoms associated with CTE. Advanced forms of CTE may be misdiagnosed as other neurodegenerative diseases such as Alzheimer's disease or even Parkinson's disease, as the clinical presentations are similar [21]. CTE patients however, rarely present with amyloid- β plaques, and the

tau pathology found in CTE is distinct from that seen in Alzheimer's disease. Neurofibrillary Tau tangles are usually distributed in the frontal and temporal cortices in close contact with cerebral vesicles or in the cerebral sulci. A CTE brain shows pronounced atrophy in the cerebral cortex, medial temporal lobe and diencephalon, and distinctly enlarged lateral ventricles [13][21].

The association of ventriculomegaly with CTE was what initially drew the Conover lab to study mTBI in a mouse model. The Conover lab focuses research on neurogenesis of the mammalian Subventricular Zone (SVZ), a region that lies along the lateral walls of the lateral ventricles [20]. The SVZ is one of two neural stem cell niches that persists into adulthood, however in response to stressors such as aging, the SVZ tends to show deterioration [28]. The SVZ is a highly organized network made up of four distinct cell types: ependymal cells, SVZ astrocytes, transitory amplifying cells, progenitor cells, and neuroblasts [20] and [25]. In the adult SVZ, proliferating radial glia cells (astrocytes) generate transient amplifying cells, which in turn give rise to neuroblasts. In the rostral migratory stream (RMS), a chain of neuroblasts from the SVZ migrates through tubes of astrocytes towards the olfactory bulb. Once the neuroblasts have reached the core of the olfactory bulb the immature neurons disengage from the RMS to differentiate into cells required for olfactory function [22]. Ependymal cells are simple ciliated epithelial cells that line the interior walls of the lateral ventricles. Junctions connect the ependymal cells together at the apical end of the cell, creating an interface between the interior of the ventricles and the rest of the brain [5]. The structure and enzymatic output of ependymal cells creates a barrier between the cerebrospinal fluid (CSF) and the brain parenchyma and assists in cerebral fluid balance, toxin metabolism and secretion. The

ependymal lining may also serve to protect the SVZ stem cell niche. Damaged ependymal cells are typically replaced by astrocytes, which form a glial scar. The glial scar has a similar morphology to the ependymal cell, however it does not contain the same cilia or protein channels that may assist in creating the CSF-brain barrier [5].

Stem cells and neuroblasts residing in the SVZ may be a potential source of new cells for neural tissue repair after mTBI. Following experimentally induced ischemic stroke in rats, several studies have shown neuroblasts from the SVZ migrate to the site of injury [17]. The majority of migrating neuroblasts that reach injured stroke regions usually remain undifferentiated, and those neuroblasts that do differentiate typically die, as they are unable to integrate into mature neuronal networks. An increase in neurogenesis in the SVZ has been shown in mouse models with induced TBI, however determining the fate of differentiated cells and their migratory patterns remains a challenge [17]. Although there is significant interest within the scientific community in neural repair, little is actually understood about how a single mTBI or multiple rmTBI might alter or change the neural stem cell niche. The initial aim of our rmTBI study was to determine whether any changes in ventricular volume could be observed over a 3-month period following rmTBI in mice. The gross ventriculomegaly seen in human CTE patients led us to hypothesize that rmTBI would result in an increase in the ventricular volume and an increase in glial scarring along the ependymal lining [28]. Damage to the lateral wall may directly impact the brain's response to physical injury and capacity for regeneration.

Recent studies have shown that microglia, the brain's resident immune cells, also play a role in SVZ neurogenesis [17] [29]. Microglia are responsible for surveying the brain for areas of damage, and are known to exhibit distinct changes in cellular

morphology and physiology in response to neuronal injury or infection [2], [18] and [23]. A healthy brain contains as many microglia as it does neurons [2]. In the resting state, microglia are identified as “ramified” with characteristically small cellular bodies and numerous thin processes extending out from the soma [18]. While the fine processes of ramified microglia are continuously moving in order to probe their environment, the soma remains relatively still [18]. Ramified microglia are rarely seen touching one another; however, they are often found in direct contact with astrocytes or neurons [2]. Microglia become “activated” in response to minute alterations in brain chemistry, including extracellular ATP, cytokines, necrotic tissue and neurotransmitters [18] [23]. Receptors on the surface of microglial membranes bind to such molecules, and signal for the cell to undergo a complex set of phenotypic changes, transforming into an “activated microglia.” Activated microglia take on a macrophage-like morphology, migrate towards sites of injury or infection, secrete numerous cytokines, phagocytose dead cells or debris and proliferate [18] [23]. An activated microglia will upregulate the expression of Iba-1, a cytoplasmic protein not expressed in any other neuronal cells, making it an ideal target for immunohistochemical assays [8].

Microglia take on one of two distinct subtypes following activation, M1 or M2 [8]. The M1 cells are characterized as having a proinflammatory profile, producing high levels of interferon-gamma (IFN- γ), tumor necrosis factor (TNF- α), interleukin-1 β (IL-1 β), IL-12 [7] [11]. M2 microglia act in opposition to M1 cells by dampening inflammation through the release of anti-inflammatory cytokines including IL-10 and transforming growth factor (TGF)- β . M2 cells also contribute to brain recovery by phagocytosing cellular debris [11].

During early postnatal development microglia are found in semi-activated states concentrated in the two neurogenic regions of the brain, the SVZ and the hippocampus [9]. The cytokines released by partially activated microglia have been shown to play a role in proliferation within the SVZ niche [27]. The function of microglia in neurogenesis is still not fully understood, however many studies illustrate a beneficial role for partially activated microglia in neurogenic environments during development or after injury [29].

When mTBI occurs, lateral acceleration and deceleration of the brain within the skull causes primary mechanical damage, resulting in the shearing of neurons, glia and blood vessels along with stretching of epithelial tissue [19]. The physical damage to tissue and breakdown of membranes leads to what is known as secondary injury, where neurotransmitters, ions and free radicals escape from dying cells inducing neuroinflammation and apoptosis [19] [30]. Responding to the accumulation of neurochemical molecules in the extracellular space, microglia become activated, taking on the M1 subtype, releasing pro-inflammatory cytokines, nitric oxide and reactive oxygen species into their environment [11]. The M1 proinflammatory cascade is important for the initiation of the immune response within the brain; however, if left unbalanced by the M2 anti-inflammatory process individuals may experience lasting mTBI symptoms or poor recovery. Repeatedly exposing the brain to mechanical injury as seen in athletes with rmTBI, may prevent microglial subpopulations from transitioning into M2 subtypes, leading to prolonged neuroinflammation and an increased risk for neurodegeneration [7].

The second aim of this study was to characterize morphological changes in microglia in mice following rmTBI. Coronal sections of brain regions susceptible to mTBI including the lateral ventricles, hippocampus, corpus callosum, and cortex were analyzed

via immunohistochemistry immediately following rmTBI and 3 months after injury. While other groups have shown mTBI can activate microglia, our study indicates that repeated head trauma in mice results in a sustained proinflammatory profile up to three months following injury. We hypothesize that these findings have broad implications for rmTBI recovery and the progression of neurodegeneration following rmTBI. Understanding the neuroinflammatory process and timeline may help researchers design successful therapeutic drugs or implement better return to play policies to avoid further injury [19].

Methods

Currently, neuroimaging techniques, such as MRI, are the best ways to study rmTBI in living humans. While there have been significant advances in radiology over the past decade, the imaging technology does not yet exist to view changes occurring within cells. In addition the costs of operating advanced MRI machines and collecting large pools of data is very expensive. This leaves post-mortem tissue analysis as the only method to study human rmTBI related brain changes at the microscopic level. When researchers examine post-mortem tissue of individuals known to have sustained multiple mTBIs throughout life, neurodegeneration is often advanced. It may also be challenging to distinguish age-related changes from those caused by rmTBI. A mouse model provides an opportunity to study the effects of rmTBI at various intervals following injury. A large body of literature indicates numerous animal models have already been developed to study TBI [10] [14] [30]; however, few models exist that accurately replicate the kind of repeated mild concussive injury an athlete may sustain on the playing field. The severity of

any TBI is highly dependent on how and where the individual was hit on the head, as well as their age, gender, and genetics [30]. The mouse model allows many of these variables to be controlled for, allowing us to develop a more generalized and simplified understanding of the primary and secondary injuries that occur following rmTBI. Models such as the fluid percussion injury (FPI) require the researcher to perform a craniotomy over the midline or parietal bone of the mouse, before a fluid pressure pulse is delivered directly to the dura, displacing the brain to induce injury to the tissue [30]. While this method can replicate the intracranial hemorrhage and neural swelling seen in human TBI, it is unrealistic and does not mimic any injury a human patient might ever sustain. The craniotomy also places the animal at an increased risk of infection, which could alter studies of microglial activation.

In 2012, Kane et al. developed a unique mouse model to specifically study rmTBI. Their model can accurately induce repeated mild concussive injuries over time and includes features that allow for free movement of the animal's head and torso during rapid acceleration and deceleration. Even after multiple hits to the head, skull fractures and intracranial bleeding are rarely observed in their mice [14]. We selected this effective and uncomplicated model for our studies of rmTBI on the lateral ventricles, SVZ and microglial activation.

The apparatus used by Kane et al to induce rmTBI is depicted in figure 1 [14]. Two Plexiglas pieces (15cm length x 9 cm width x 23 cm depth) make up the frame of the apparatus. An aluminum foil sheet is used to create a bridge across the top of the Plexiglas frame. A sponge cushion (15 cm x 9 cm x 13 cm) is placed 10 cm below the aluminum foil bridge. After being anesthetized with isoflurane, the mouse is placed on the aluminum foil

bridge with its head directly under a PVC guide tube (20 mm diameter x 1.5 m length). A 95g solid brass weight is released 40 mm from the bottom of the PVC tube, and falls directly onto the midline of the mouse's head between the ears. When the weight makes contact with the mouse the aluminum foil sheet tears apart, allowing the mouse to free fall and perform a 180° flip. The mouse will land supine on the cushion 10 cm below the initial resting point. Sham control mice receive the same dose of isoflurane as experimental mice and are positioned on the apparatus, but do not receive an impact or fall through the aluminum foil bridge.

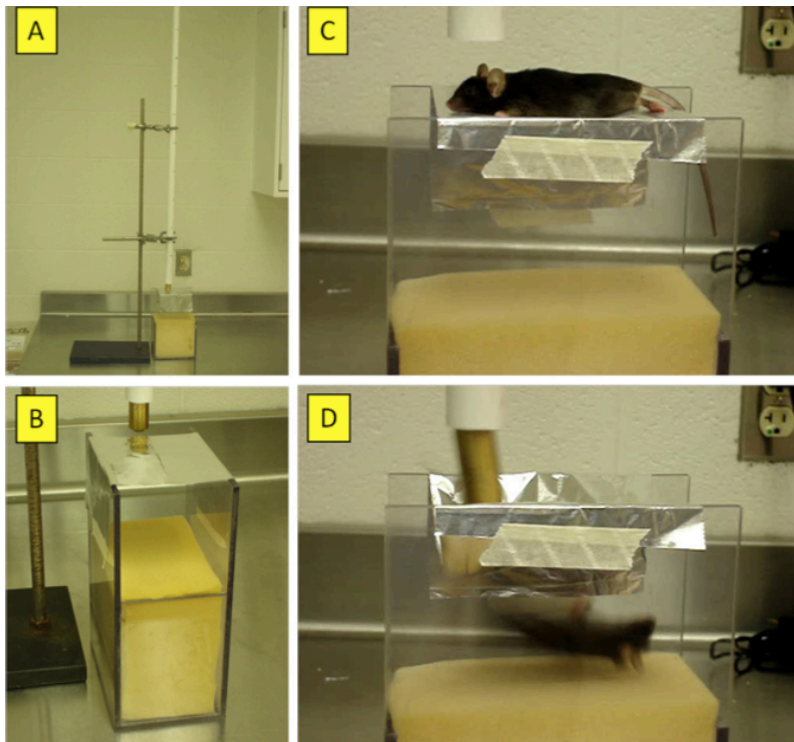


Figure 1. rmTBI apparatus (A) The entire apparatus including the (20 mm x 1.5 m) PVC delivery tube and plexiglas frame. (B) Aluminum foil bridge is pictured over the Plexiglas frame. (C) The mouse is shown in the initial position with head directly below the PCV delivery tube. (D) Following impact with 95 g weight the mouse breaks through the aluminum foil bridge, completes 180° flip falling onto the cushion 10 cm below [14].

We designed two different rmTBI studies (Figure 2) that were carried out on the Kane et al. group's apparatus at Wayne State University [14]. In the first study C57 mice received five hits with a 95 g weight over three days, with hits being carried out in the morning and the evening. Following the fifth hit, mice received an injection of

bromodeoxyuridine (BrdU) to mark cells undergoing DNA replication. Mice were then sacrificed at four time points following the last impact: 2-hours, 2-weeks, 1-month and three-months post injury. In our second study mice received an initial five hits over 3 days, then underwent a resting period of two weeks. Following the resting period experimental mice were subjected to an additional round of five hits over three days then a BrdU injection immediately after the last impact. Mice from the second study were sacrificed at three time points after the final injury: 2-hours, 1-month and 3-months following injury.

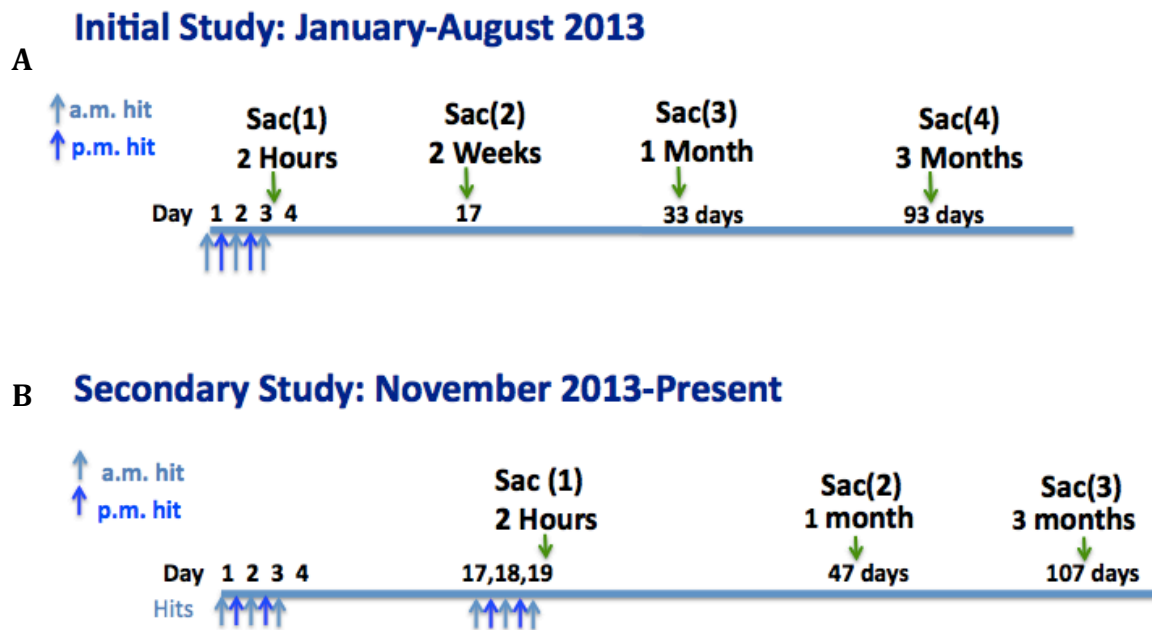


Figure 2. rmTBI study outline (A) Depicts the outline for the initial rmTBI used to complete ventricular volume assessments. **(B)** Shows the second rmTBI study outline employed in the microglial activation studies.

Researchers from Wayne State University were responsible for inducing rmTBI and sacrificing mice according to the established time line (Figure 2). Mice were sacrificed via cardiac perfusion with saline and 4% PFA followed by excision of the entire brain.

Brains were shipped in PBS to University of Connecticut where members of the Conover Lab carried out all further analysis.

Immunohistochemistry

Control and experimental brains were processed and analyzed using the same methodology. For both the ventricular volume analysis and microglial activation study, brains were embedded in agarose, mounted on a vibratome (VT-1000S; Leica), and then sectioned into 50 μm coronal slices. Sections were blocked in 10% Horse Serum (Invitrogen) in PBS/0.1% Triton X-100 for one hour. For the ventricular volume studies, brains were then incubated with rat anti-BrdU, mouse anti-GFAP (Abcam) and goat anti-Aquaporin-4 primary antibodies. The BrdU antibody marks cells that underwent division at the time of the IP BrdU injection. GFAP (Glial Fibrillary Acidic Protein) is a marker for reactive astrocytes, and Aquaporin-4 (AQP4) marks water channels located on the basal-lateral membrane of ependymal cells. Before applying the BrdU primary antibody the sections were acid-fast treated.

For the microglial activation study rat anti-GFAP (Abcam), rabbit anti-Iba-1 (Wako) and mouse anti-Doublecortin (Abcam) were used as primary antibodies. Iba-1 is an intracellular calcium binding protein that is highly expressed in activated microglia, and doublecortin (DCX) is a marker for neuroblasts.

After 24 hours incubation with primary antibodies at 4°C, sections were rinsed three times in PBS before the addition of corresponding Alexa Fluor dye-conjugated secondary antibodies (Invitrogen) to the tissue for 1 hour at room temperature. Following secondary antibody removal the tissue was rinsed two times, DAPI nuclear stain was added to the tissue, then all sections were rinsed 3 additional times in PBS. Sections were

mounted onto glass slides then coverslipped with Aqua-Poly/Mount (Polysciences) and allowed to dry completely.

Ventricular Volume

To determine the volume of the lateral ventricles in control and rmTBI mice, the lateral ventricles were viewed on a Carl Zeiss Axio Imager M2 Microscope with Apotome (Carl Zeiss) at 10x then traced using StereoInvestigator software. Approximately 38 50 μ M sections were traced to make up the entire lateral ventricle. Following tracing the sections were uploaded and stacked in the program Neurolucida Explorer, to create a 3-D model of the lateral ventricles. Once the 3-D model was complete, the software was able to calculate consistently accurate ventricular volumes. Figure 3 depicts stacked lateral ventricle sections and a 3-D reconstruction of the brain and lateral ventricles. A students t-test $p < .05$, was performed on volume data and SEM error bars were added.

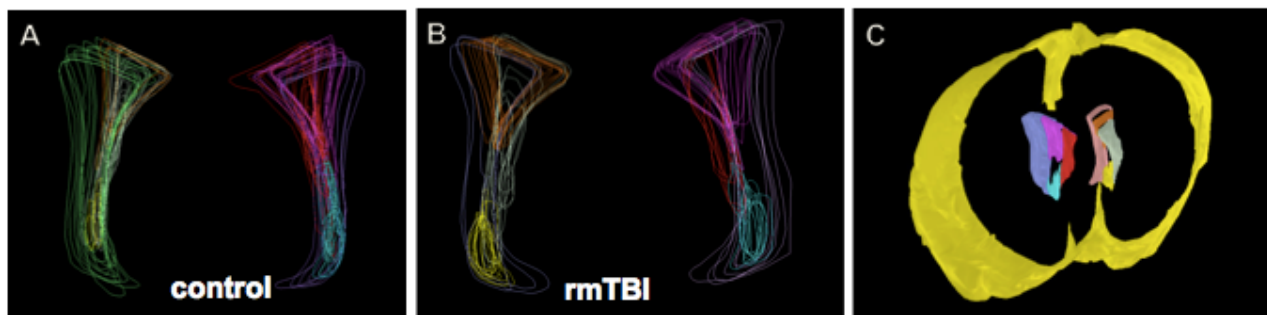


Figure 3. Lateral Ventricle Tracing and 3D Reconstruction Stacked lateral ventricles from a (A) control mouse and (B) rmTBI mouse in the Neurolucida Explorer program. (C) 3-D reconstruction of stacked coronal showing the lateral ventricles and whole brain. Lateral ventricle volumes were calculated from 3-D reconstruction.

Imaging of Microglia

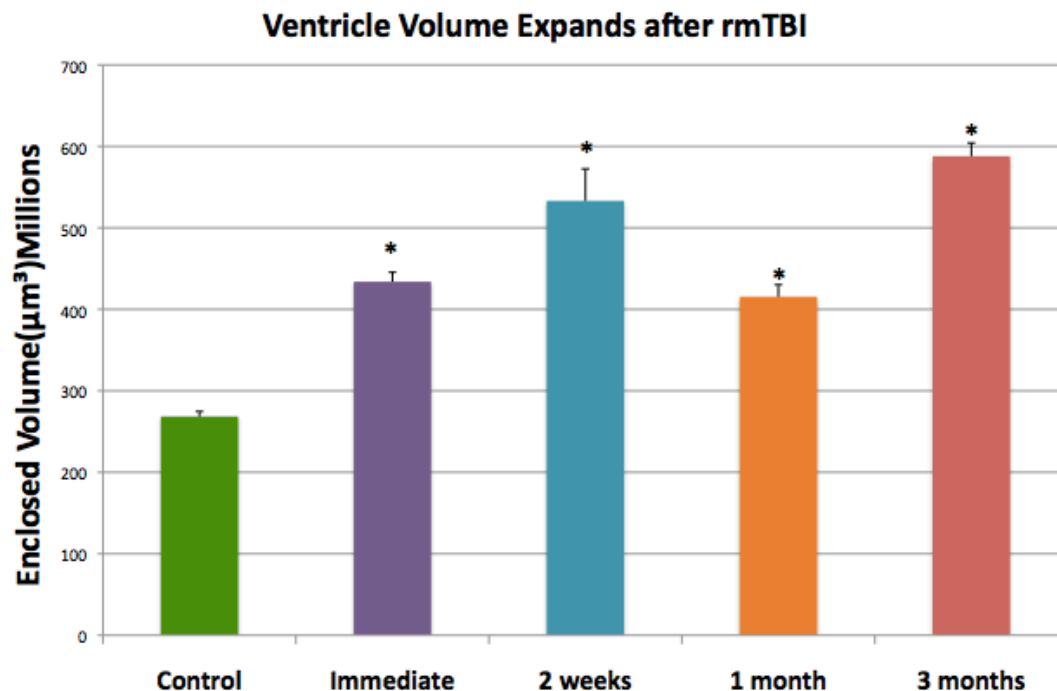
Following immunohistochemical preparation, Iba-1⁺ microglia were imaged at 10x on the Carl Zeiss Axio Imager M2 Microscope with Apotome (Carl Zeiss) using

Hamamatsu ORCA-R2 digital camera C10600 and at 40x on a Leica TCS SP2 confocal laserscan microscope. The gain and light exposure were kept constant throughout all imaging.

Results

Expansion in the lateral ventricles following rmTBI

Reconstruction of the lateral ventricles from mice in the initial rmTBI study revealed a gradual increase in lateral ventricular volume during a 3-month interval in rmTBI mice over control mice. We noticed the greatest volume expansion in 3-month mice and a slight decrease in volume from the 2-week to 1-month time points.



Microglial Activation Following rmTBI

Figure 4. Lateral ventricular volume expands after rmTBI. Immediately following the 5 hits over 3 days rmTBI model, mice experience ventricular expansion. The lateral ventricles continued to expand over the course of the 3-month interval to the highest volume recorded. All four experimental groups show significance over control (* $p < 0.05$, students t-test, SEM error Bars).

Four brain regions were selected to examine the effect of rmTBI on microglial activation. The lateral ventricles were selected due their close association with the neurogenic SVZ niche. The hippocampus was examined next as it is the other site of neurogenesis in the brain. The corpus callosum was chosen for analysis, as it is made up of long white matter tracts, which are highly susceptible to tearing as a result of the lateral acceleration/deceleration and torsional brain movement occurring during rmTBI. Microglia in the cortex by the site of impact were also analyzed for changes in morphology.

Changes in microglia morphology and activation varied in different brain regions and was altered over time. Along the border of the lateral ventricles (Figure 5), an increase in soma size can be observed in mice sacrificed 2-hours following rmTBI. Both increases in soma size and cell process thickness are noticeable in rmTBI mice 3-months after injury. In the hippocampus the rmTBI mice sacrificed 3-months after injury had more microglia with larger cell bodies and thicker processes than 2-hour rmTBI mice or either control mouse. In the corpus callosum microglia appear more frequently in the activated phenotype 2-hours after rmTBI than any other group. Within the cortex of rmTBI mice sacrificed 2-hours following injury, microglia can be seen clustering together in direct contact with one another. While this is highly uncommon among ramified resting microglia, it has been observed in activated microglia. At 3-months post injury the microglia in the cortex have much larger cellular bodies than age matched controls.

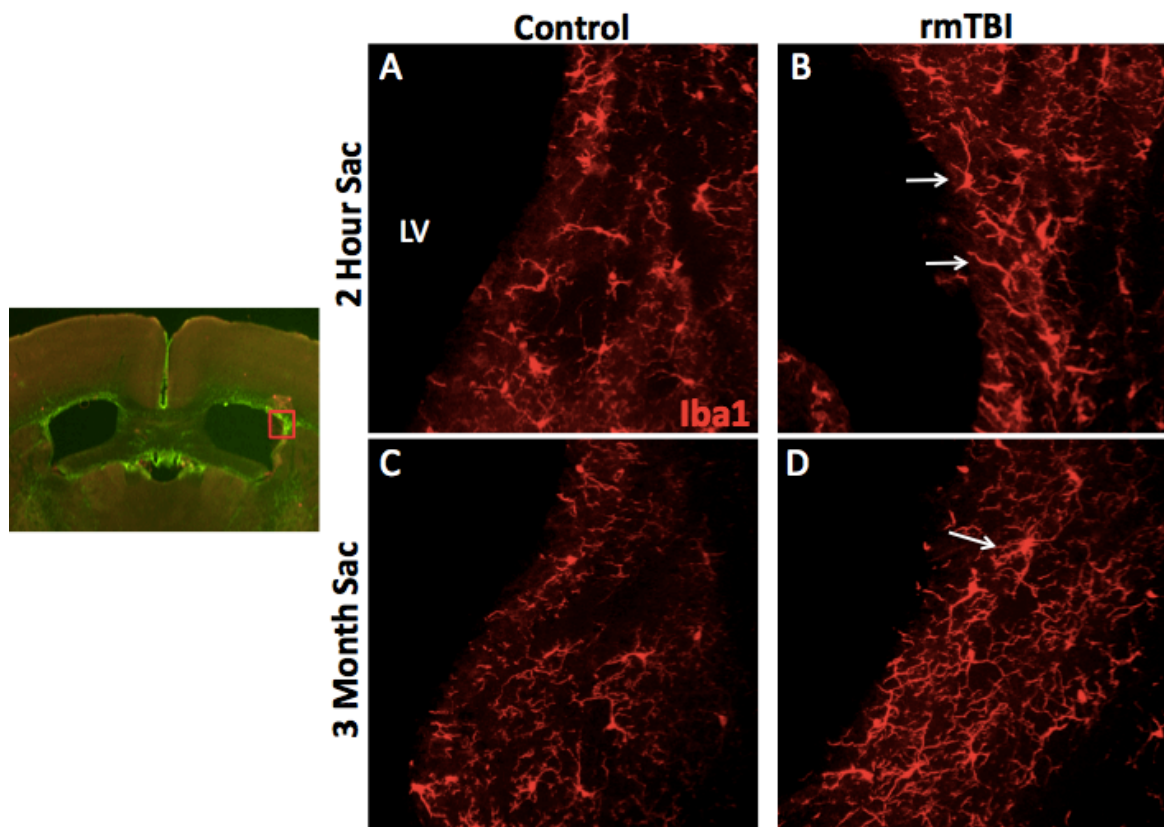


Figure 5. Lateral Ventricle microglial expression. Activated microglia express Iba1. rmTBI microglia show larger cell bodies and thicker cell processes in rmTBI **(B)** 2-hours post injury and **(D)** 3-month post injury mice. Control mice **(A and C)** appear similar to one another.

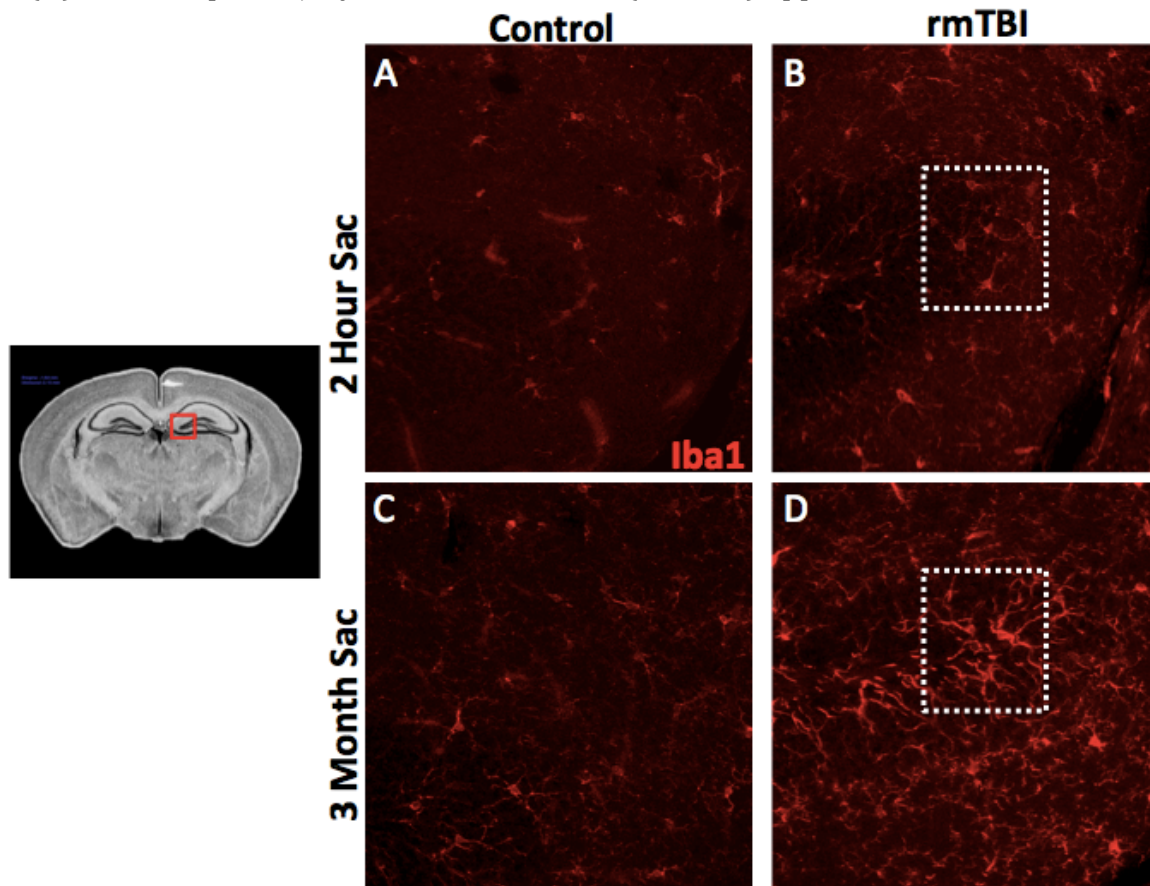


Figure 6. Activated microglia in the dentate gyrus of the hippocampus. An increase in the number of microglia can be seen in the **(B)** 2-hour rmTBI over the **(A)** 2-hour control mouse. **(D)** 3-month rmTBI microglia exhibit distinct changes to morphology with large cell bodies and thick cell processes

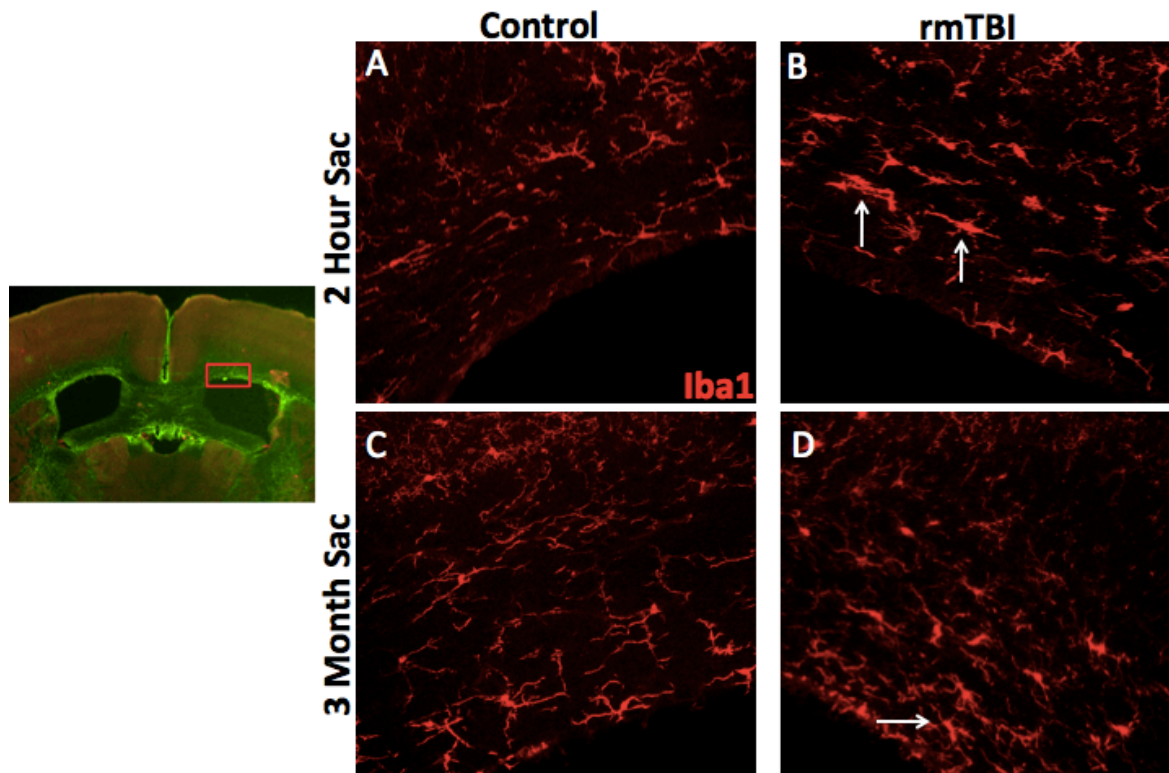


Figure 7. Corpus callosum expression of activated microglia. More activated microglia are apparent in the (B) 2-hour rmTBI mouse than the (A) control 2-hour mouse. (D) at 3-months post rmTBI microglia remain in an activated morphology compared to age matched control (C).

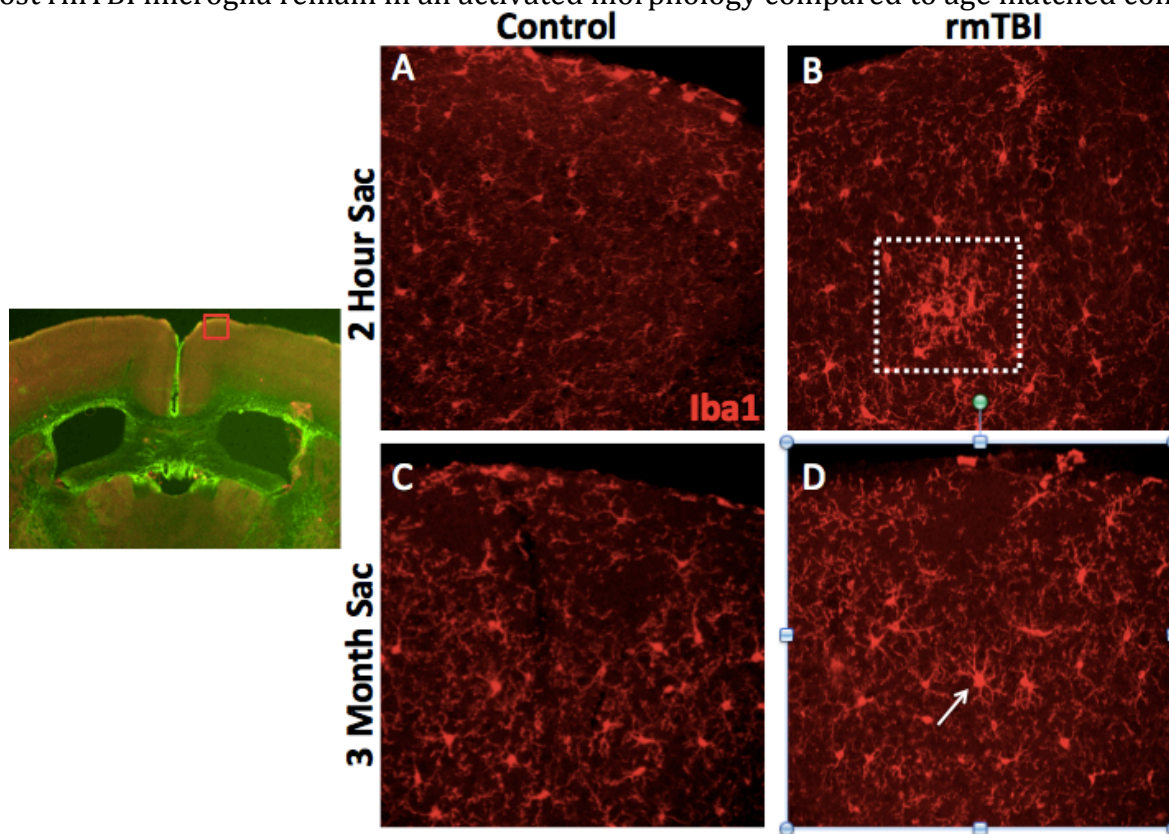


Figure 8. Cortex expression of activated microglia. Activated microglia express Iba1. The dotted box in (B) surrounds clustering activated microglia. (D) 3-months after rmTBI microglia remain activated in the cortex with large cell bodies compared to those seen in age matched

Discussion

The objective of our rmTBI mouse study was initially to identify changes in ventricular volume following repeated head injury. We hypothesize that alteration in ventricular size or ependymal integrity could have negative implications for the SVZ stem cell niche that lies along the lateral wall of the lateral ventricles. Stretching of the lateral ventricles can lead to ependymal cell tearing, inducing glial scarring along the lateral wall [28]. Glial scarring along this region may compromise the brain's ability to remove toxic molecules from the parenchyma into the CSF, exacerbating neurodegeneration and possibly increasing neuroinflammation. The data from our ventricular tracing experiment shows that following rmTBI lateral ventricular volume gradually increases over 3-months. While we were unsurprised that there was expansion of the ventricles immediately following rmTBI, as this is seen in human TBI patients, we did not expect the expansion to continue throughout the entire 3-month period. This suggests that physicians caring for rmTBI patients should pay close attention to alterations in ventricular volume and size as changes could have effects on global neuronal health and recovery. As the SVZ lies directly along the lateral ventricles neurogenesis may also be impaired by glial scarring and ventriculomegaly reducing the capacity for regeneration.

After observing a gradual increase in ventricular volume over 3-months in our initial rmTBI mouse model study, we moved our focus towards the neuroinflammatory process associated with rmTBI. We aimed to characterize microglial activation following rmTBI using confocal microscopy. Our rmTBI mouse model, designed by Kane et al., was specifically selected for its accurate replication of the rapid acceleration and deceleration of the brain that commonly occurs in human rmTBI, such as in a sports concussion. These

forces along with torsional rotation of the brain within the skull are thought to contribute to the primary mechanical injuries of rmTBI including tearing and damage to axons, cell bodies and neuronal networks. The leaking of intracellular molecules such as neurotransmitters and reactive oxygen species into the extracellular space induces secondary injury, leading to neuroinflammation and prolonged rmTBI symptoms. The activation of microglia is known to play a central role in the neuroinflammatory pathway, with M1 activated subtypes releasing pro-inflammatory cytokines and M2 microglia counterbalancing by releasing anti-inflammatory molecules into the brain [7] and [8].

To gain a better understanding of the distribution of microglia throughout the brain following rmTBI and the timeline of activation, we examined microglia in four different brain regions: the lateral wall of the lateral ventricle, the hippocampus, the corpus callosum and the cortex. We found that 2-hours after rmTBI noticeable changes in microglial morphology could be observed in the lateral ventricle wall, the corpus callosum and cortex. Cell processes were thicker and cell bodies increased in size. At 3-months following rmTBI all experimental mice showed increased microglial activation over age-matched controls. The hippocampus showed the greatest microglial activation compared to age matched control. Observing microglia in an activated state 3-months following rmTBI indicates the inflammatory process persists long after injury. If the activated microglia are remaining in an M1 subtype, pro-inflammatory cytokines such as IL-1 β that retard recovery and promote rmTBI symptoms may still be modulating the brains environment [7]. Recently, Fenn et al. showed mice with induced mTBI respond by releasing significantly more pro-inflammatory molecules when challenged with an IP lipopolysaccharide (LPS) injection, 30-days after mTBI injury, over control sham mice.

Following the LPS injection, mTBI mice took much longer to return to their baseline behavioral scores than control mice [7]. This study along with our own research indicates that prolonged microglial activation can occur following rmTBI, and that the activated microglia can prevent the brain from effectively responding to infection or injury for a significant period of time following rmTBI. Further studies on the M1/M2 subtypes of microglia present in the brain following rmTBI may be helpful in elucidating the neuroinflammatory pathways and generating timelines for therapeutics.

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